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Award Number: W81XWH-08-1-0374

TITLE: Genetic Manipulation of Mammary Stem Cells to Reconcile Tumor Stem Cell Theory with Breast Cancer Heterogeneity

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REPORT DATE: July 2009

TYPE OF REPORT: annual summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

x Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-07-2009	Annual Summary	1 Jul 2008 - 30 Jun 2009

4. TITLE AND SUBTITLE
Genetic Manipulation of Mammary Stem Cells to Reconcile Tumor Stem Cell Theory with Breast Cancer Heterogeneity

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0374

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Justine Hutchinson

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Dartmouth College
Hanover, NH 03755

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

No abstract provided.

15. SUBJECT TERMS
No subject terms provided.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	10	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

This report serves as a progress report on the first year of my training grant. I have just completed my fourth year as a graduate student in Dartmouth Medical School's Department of Pharmacology and Toxicology, part of the Program in Experimental Molecular Medicine (PEMM). Dr. James DiRenzo serves as my mentor and chair of my thesis committee, and also directs the lab where I work, hereafter referred to as "the DiRenzo lab".

BODY

Training Tasks
I completed all coursework required for the PhD degree, including courses in medical pharmacology and cancer biology, and passed the so-called "qualifying exam," which consists of writing a grant proposal and defending it to a panel of faculty. Senior members of the DiRenzo lab have trained me extensively in mouse handling, histology and tumor pathology. I was also trained to isolate mammary epithelial cell populations by fluorescence activated cell sorting (FACS), and have developed some of my own protocols for FACS-based experiments. Dr. DiRenzo has also trained me in the surgical technique of transplanting mammary epithelial cells into cleared mammary fat pads.

Meetings Attended
The National Symposium for the Advancement of Women in Science (NSAWS), Harvard University, February 6-7, 2009
2009 Dartmouth Breast Cancer Symposium, June 19, 2009

Seminars Given
Mouse Models Made Easy: Studying Cell Fate in a Classic Mouse Model of Human Breast Cancer, Dartmouth Medical School, June 3, 2009. (Find images of the slides for this presentation attached in the appendix.)

Research Tasks

Aim 1: Lentiviral mediated gene targeting of self-renewing and non-self-renewing mammary epithelial populations.

I made high-titer lentivirus-containing media by transfecting 293T cells simultaneously with separate plasmids coding from the viral proteins GAG and POL, as well as the lentiviral overexpression vector pLOVE bearing either the gene for green fluorescent protein (GFP) or the N-myc oncogene. An infection efficiency near 100% was achieved in mammary epithelial cell lines by addition of polybreen to viral stocks before infection.

In vitro studies were done in Immortialized Mammary Epithelial Cells (IMEC), a human cell line established by my mentor James DiRenzo some years ago by telomerase-

mediated immortalization of cells obtained from reduction mammoplasties. Protein expression profiles reveal IMECs to resemble basal epithelium. Functionally, they have characteristics of both stem and progenitor cells. Lentiviral expression of N-myc in IMEC cells grown in a monolayer triggered apoptosis in a large proportion of the cells; remaining cells failed to proliferate. By contrast, lentiviral expression of N-myc in IMEC cells grown in the low-binding mammosphere assay did not promote cell death; the rate of mammosphere formation from the N-myc expressing cells was comparable to the GFP control, but the spheres formed were slightly larger and had a smoother, rounder shape, suggesting more symmetrical mitotic events than asymmetrical ones.

Infection efficiency of primary mouse mammary epithelial cells was lower, but GFP-positive cells were clearly visible in low-binding cultures of isolated primary mammary epithelial cell populations after several days. From there we proceeded to the transplantation of lentiviral-infected primary cells. Expression of GFP was not detectable within the epithelial cells in the recipient glands after 10 weeks. There are several potential explanations for this, the most likely being the very low infection rate of the primary cells used for the transplantation. We have acquired a c-myc gene-bearing retrovirus from Dr. Michael Cole at Dartmouth College to be used in conjunction with an irradiated feeder layer as one potential solution to this problem. We have also experimented with adenoviral infection of primary cells, but expression was not detectable in transplant recipients.

Aim 2: Transgenic targeting of oncogenic alleles into mammary stem cells.

ΔN-p63-eGFPcre and K14-eGFPcre transgenes were constructed as described in the original grant proposal. When these transgenes were transfected into mammary epithelial cell lines, limited GFP expression was observed, consistent with the concept of targeting a self-renewing, stem-cell-like subpopulation. The transgenic mouse lines we created using these transgenes, by contrast, did not have a detectable subpopulation expressing GFP protein either when whole-mounted glands were observed under the fluorescence dissecting microscope, by IHC of fixed tissue, or when the dissociated epithelial cells were evaluated by flow cytometry.

Since the goal of this project was to determine whether the ΔN-p63 or K14 promoters could be used to target oncogene expression to distinct subpopulations, we proceeded to determine whether these transgenes were being transcribed in the different epithelial subpopulations. Using FACS based on surface markers CD24 and CD29, we isolated stem-cell-enriched, luminal progenitor cell and stromal cell subpopulations from transgene-bearing and wild-type siblings. RNA was isolated from these cells and analyzed by reverse transcription-polymerase chain reaction analysis (RT-PCR). GFP mRNA was not found in ΔN-p63-eGFPcre mammary gland. In the K14-eGFPcre mouse, GFP mRNA was detected in both the stem cell and progenitor cell fractions.

Another transgene described in the original proposal, the ΔN-p63 promoter fused to a

floxed (LoxP-flanked) stopper sequence and cDNA for c-myc (ΔN-p63-LSL-c-myc), was tested in vitro in IMECs, because they have a uniformly high expression of the ΔN-p63 protein. First, the transgene was transfected into IMECs, then cells with stable integration of the transgene were selected by exploiting a neomycin resistance gene. The cells were then infected with an adenovirus bearing a gene for Cre-recombinase. Successful genomic recombination was detected by polymerase chain reaction (PCR) analysis of genomic DNA. RT-PCR analysis was used to determine expression of transgenic c-myc RNA. No such RNA was detected, so that transgene was abandoned.

Seeking another method to determine the effects of c-myc expression on the mammary stem cell in vivo, we obtained an MMTV-myc transgenic mouse. The MMTV-myc mouse is a classic model of human breast cancer, with the following characteristics:

- MYC expression is specific to the mammary epithelium, but NOT dependent on lactation, and not during early development.
- Nulliparous mice have low rates of mammary tumorigenesis.
- Virtually 100% of parous mice develop mammary tumors. (Reviewed in Hanahan, 2007).
- Mice exhibit premature lactation during pregnanacy, and the involution response if the mammary gland to milk stasis is not compromised. (Blakely, 2005).

Transplantation of stem cells from mature MMTV-myc mice and wild-type siblings into wild-type recipients allowed for the expression of MYC in a mitotically active mature stem cell. Analysis of the resulting grafts revealed an apparent phenotype, but an inconsistent one. Future experiments that could yield more interpretable results include limiting dilution transplantations, and analysis at progressive timepoints. Ongoing experiments to determine the quiescence of mature MMTV-myc stem cells include *in vitro* low-binding culture of isolated populations and *in vivo* long term labeling experiments to assess BrdU retention.

Since pregnancy is the primary risk for tumorigenesis in MMTV-myc mice, and pregnancy and transplantation both cause stem cells to exit quiescence, longer-term transplantation experiment to assess tumorigenesis is also underway.

KEY RESEARCH ACCOMPLISHMENTS

1. The successful isolation and transplantation of an enriched population of mammary stem cells.
2. The ruling out of the K14 and ΔN-p63 promoter as efficient means of targeting specific epithelial subpopulations in transgenic mice.

REPORTABLE OUTCOMES

There are no reportable outcomes at this time.

CONCLUSION

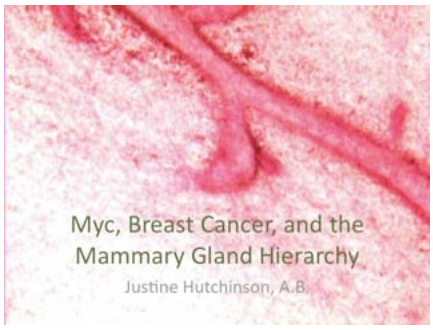
Although our research has not gone exactly according to plan, I have made significant progress, both toward my technical training, and toward fulfilling the aims that I laid out in the proposal for this grant. In the next year, I hope to continue my progress with the evaluation of oncogenic activity of myc in epithelial subpopulations, to write a paper, and to begin to attend larger scientific meetings.

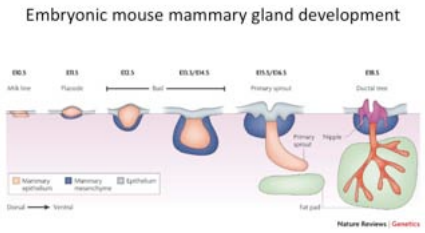
REFERENCES

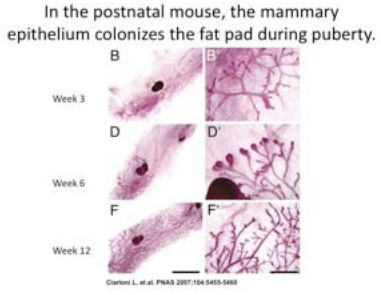
1. Hanahan D, Wagner EF, and Palmiter RD. (2007). *Genes & Dev*., **21**, 2258-2270.
2. Blakely CM, Sintasath L, D'Cruz CM, Hahn KT, Dugan KD, Belka GK and Chodosh LA. (2005). *Development*, **132**, 1147-1160.

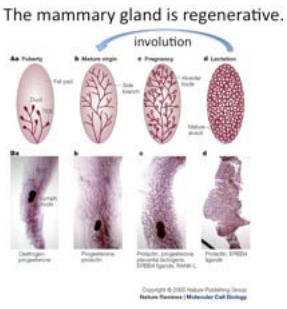
APPENDICES

Appendix 1
Slides from the seminar entitled *Mouse Models Made Easy: Studying Cell Fate in a Classic Mouse Model of Human Breast Cancer*, Presented by Justine Hutchinson at Dartmouth Medical School on June 3, 2009.









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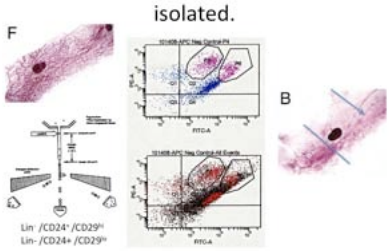
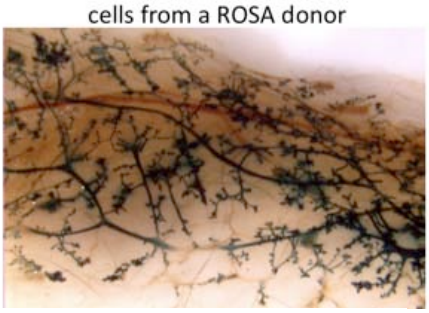
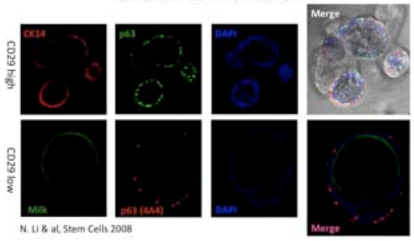
Epithelial subpopulations can be isolated.



Outgrowth of transplanted CD29^{high} cells from a ROSA donor

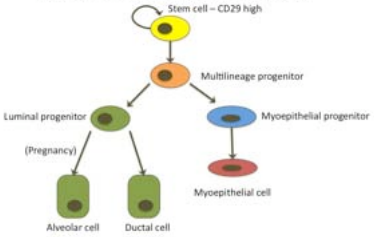


Subpopulations produce different structures in culture.

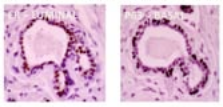


N. Li & al, Stem Cells 2008

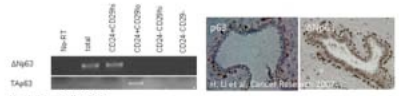
Within the CD24+/CD29low progenitor population, there are distinct luminal and myoepithelial progenitors.



Mammary epithelium has distinct luminal and basal layers.

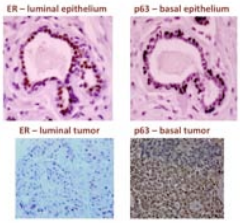


The basal epithelium contains the regenerative populations.

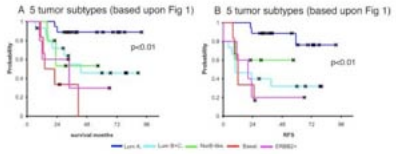


Na Li, Stem Cells 2008

Just as there are distinct basal and luminal epithelia, there are distinct basal and luminal tumors.



Mammary tumor subtypes have:
- characteristic gene expression signatures
- differing prognoses
- predictable responses to certain drugs



Sorlie et al, PNAS 2002

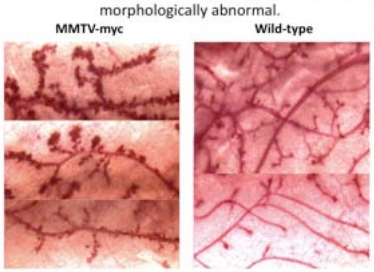
MYC and cancer

- MYC is a proto-oncogene
- MYC has a host of functions: Replication, growth, metabolism, differentiation, apoptosis
- Instances of amplification or overexpression of MYC have been reported for virtually all malignancies.
- Small molecule inhibitors of the MYC protein are in development.
- MYC expression is elevated in approximately 70% of human breast cancers.

The MMTV-myc mouse is a classic model of breast cancer.

- MYC expression is specific to the mammary-epithelium, but NOT dependent on lactation.
-- Low levels of spontaneous mammary tumors in virgin miice.
-- Virtually 100% occurrence of mammary tumors in parous mice.
-- Etc.

Mammary epithelium from virgin MMTV-myc mice is morphologically abnormal.



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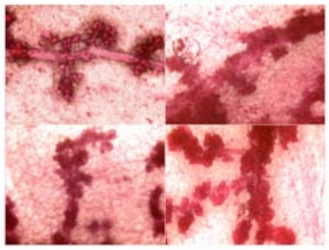
Abnormalites in MMTV-myc epithelium do not appear to include ductal occlusion.



Sections of glands from mature virgins

- Showing NO signs of ductal occlusion (sort of the opposite, actually)
- Premature alveolar formation
- I've seen these slides, and hope to take pictures today or tomorrow.

Sections from precancerous glands

- I have two normal-looking glands from a mouse that had a tumor in another gland. I hope to have some of these images by the end of the week. I expect to find ductal occlusion, carcinoma in situ, etc.

MYC and other mouse models of breast cancer

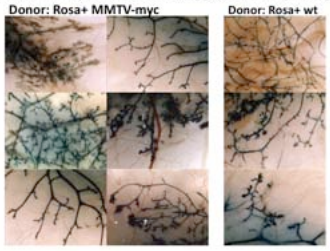
Important findings from the models that have inducible MG-specific MYC expression:

-The majority of MYC-induced mammary tumors continue to grow in the absence of MYC overexpression. Boxer et al., Cancer Cell 2004

-Ectopic MYC expression during pregnancy causes premature lactation; tied to a 3-day window mid pregnancy. Blakely et al, Development 2005.

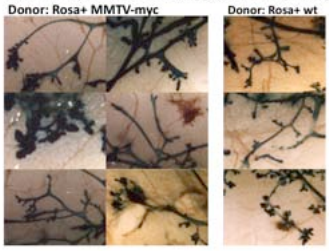
Wholemounts of MGs transplanted with epithelium from MMTV-myc mice show exaggerated morphologies

Donor: Rosa+ MMTV-myc | Donor: Rosa+ wt



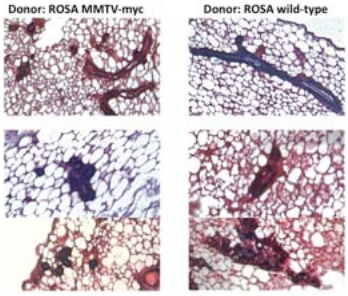
Wholemounts of MGs transplanted with epithelium from MMTV-myc mice show exaggerated morphologies

Donor: Rosa+ MMTV-myc | Donor: Rosa+ wt



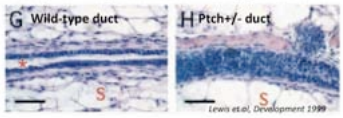
Ducts from MMTV-myc donors are not occluded.

Donor: ROSA MMTV-myc | Donor: ROSA wild-type



Outgrowths from Ptch+/- donors exhibit hyperramification.

Ptch+/- mammary ducts are occluded by epithelial cells.



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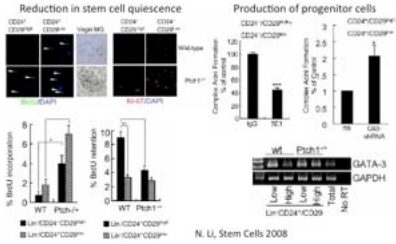
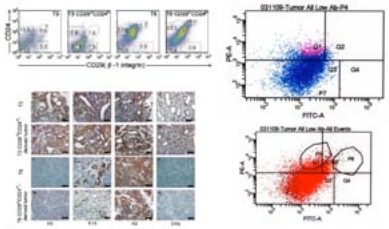
Hh signaling reduces stem cell quiescence, and increases the production of progenitor cells.



N. Li, Stem Cells 2008

Mammary tumors from P53-/- mice have a CD24+/ CD29high tumor-initiating cell population; do tumors from MMTV-myc mice?



M. Zhang et al, Cancer Research 2008

Working hypotheses about MYC, experiments in progress and future directions

Because both the Ptch+/- and MMTV-myc models share the hyper-ramification phenotype, and because a stem cell quiescence defect is believed to be responsible for that phenotype the Ptch+/- model, we are looking for such a defect in the MMTV-myc model.

Future directions:

Differences between the two models should also be explored.

Thank you:

DiRenzo Lab	Dartmouth	Thesis Committee
Dr. Jim DiRenzo	Ann Lavanway	Dr. David Robbins
Sierra Kent	Gary Ward	Dr. Sarah Freemantle
Pratima Cherukuri	The Vail animal techs	Dr. Charles Cole
Dr. Hua Li		